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[Designation of Document] specification

[Title of the Invention] SOLID SUPPORT HAVING ELECTROSTATIC LAYER AND APPLICATION THEREOF

[Claims]

The solid support according to claim 1, wherein the surface of the substrate is surface-treated with at least one kind selected from diamond, a soft diamond, a carbonaceous matter and carbide.

[Claim 3]

The solid support according to claim 1 or 2, wherein the electrostatic layer comprises an amino group-containing compound which does not covalently bind to the substrate.

[Claim 4]

The solid support according to claim 1 or 2, wherein the electrostatic layer is composed of an amino group-containing compound which covalently binds to the substrate and the amino group-containing compound has an amino group at the terminus to which the substrate does not bind.

[Claim 5]

The solid support according to any one of claims 1 to 3, which is obtained by depositing a compound having an unsubstituted or monosubstituted amino group and a carbon compound on the substrate and then introducing a functional group capable of covalently binding to a nucleic acid molecule.

[Claim 6]

The solid support according to claim 6, wherein the compound having an unsubstituted or monosubstituted amino group is polyarylamine.

[Claim 8]

The solid support according to any one of claims 1 to 7, wherein the nucleic acid molecule is DNA.

[Claim 9]

An immobilized nucleic acid molecule, which comprises a nucleic acid molecule immobilized on the solid support according to any one of claims 1 to 8.

[Claim 10]

A method of producing a solid support characterized by depositing a compound having an unsubstituted or monosubstituted amino group and a carbon compound on the substrate and then introducing a functional group capable of covalently binding

to a nucleic acid molecule.

[Claim 11]

A method of producing a solid support characterized by dipping the substrate in a solution containing a compound having an unsubstituted or monosubstituted amino group and then introducing a functional group capable of covalently binding

[Claim 13]

A method of detecting a nucleic acid molecule, which comprises immobilizing a primer on a solid support according to any one of claims 1 to 8, hybridizing a nucleic acid molecule to the primer, extending a nucleic acid molecule complementary to the nucleic acid molecule in the presence of a labeled nucleic acid and reading a signal derived from the labeled nucleic acid incorporated into the complementary nucleic acid molecule.

[Claim 14]

A method of amplifying a nucleic acid molecule by immobilizing a primer on a solid support according to any one of claims 1 to 8, hybridizing a nucleic acid molecule to the primer and subjecting it to PCR reaction.

[Claim 15]

A method of amplifying DNA by immobilizing a primer on

a solid support according to any one of claims 1 to 8, hybridizing DNA to the primer and performing reaction with a strand-displacing DNA polymerase.

[Claim 16]

The method according to claim 13, which further comprises the step of amplifying the nucleic acid molecule after

[Detailed Explanation of the Invention]

[0001]

[Technical Field to which the Invention Belongs]

The present invention relates to a support for immobilizing DNA or the like and an immobilized nucleic acid molecule.

[0003]

The polymerase chain reaction (PCR) is a method in which a target DNA is flanked by a pair of primers, a DNA polymerase is allowed to act on it repeatedly, whereby the region flanked by the primers can be continuously amplified.

[0004]

By PCR, only the target sequence can be substantially correctly amplified to produce a large number of copies, further an efficient amplification is possible in a short time, therefore, PCR is widely used for a variety of studies, assays, tests and the like in biochemical, medical fields, etc., at present.

[0005]

It has been conventionally assumed that the principle of PCR is to control temperature, and the reaction is performed by repeating heating and cooling procedures (thermal cycle).

More specifically, after denaturing a double-stranded DNA molecule, which is the target for amplification, into complementary single strands at a high temperature, annealing a primer, which has been selected to be complementary to a part of the DNA, to the strand by cooling, and extending the DNA to the downstream of the primer with a DNA polymerase by heating

in order to disrupt the hydrogen bond of the double-stranded DNA, 2) subsequently, lowering the temperature of the sample to 45C° in order to recombine the DNA to a primer for replication, 3) further, raising the temperature of the sample to 74C° in order to replicate the DNA by extending the primer with a heat-resistant polymerase, a number of times. In such a DNA amplification reaction, the foregoing thermal cycle was carried out by putting a sample into a container made of a synthetic resin or the like, and accommodating this container into an aluminum block.

[0007]

However, the foregoing thermal cycle consumed a lot of time, and it took several hours to obtain a required amount of DNA. In addition, when the reaction is carried out by controlling the temperature by heating and cooling, there is a limit on

changing the temperature in an instant, and changeover of each step cannot be performed smoothly, whereby the accuracy of the nucleotide sequence to be amplified may be affected, or DNA other than the target may be replicated in some cases. Further, in order to change the temperature rapidly, a special apparatus or technique is needed, therefore there are an economic problem

chemically modified layer having a functional group capable of covalently binding to a nucleic acid molecule sequentially has been developed (for example, see patent literatures 3 to 5).

[0009]

However, the amount of DNA immobilized on the foregoing solid support to DNA and the bond strength to DNA are not always sufficient, therefore, the emergence of a solid support capable of immobilizing DNA in a higher proportion and with a higher bond strength to DNA has been awaited.

[0010]

The information on prior art literature relating to the invention of this application is as follows.

[Patent Literature 1]

JP-A-7-75544

[Patent Literature 2]

JP-A-7-303469

[Patent Literature 3]

WO00/22108

[Patent Literature 4]

WO/02 12891

[Patent Literature 5]

acid molecules.

[0012]

[Means for solving the Problem]

The present inventors carried out intensive studies in order to solve the foregoing problems, and as a result, found out that the immobilized amount of nucleic acid molecules and the bond strength to nucleic acid molecules are significantly improved by further providing an electrostatic layer for electrostatically attracting nucleic acid molecules on a solid support having a functional group capable of covalently binding to a nucleic acid molecule on a substrate, thereby accomplishing the present invention.

[0013]

That is, the present invention includes the following inventions.

(1) A solid support having an electrostatic layer for electrostatically attracting a nucleic acid molecule and a functional group capable of covalently binding to a nucleic acid molecule on a substrate.

(2) The solid support according to the foregoing (1), in which the surface of the substrate is surface-treated with

(4) The solid support according to the foregoing (1) or (2), in which the electrostatic layer is composed of an amino group-containing compound which covalently binds to the substrate and the amino group-containing compound has an amino group at the terminus to which the substrate does not bind.

(5) The solid support according to any one of the foregoing (1) to (3), which is obtained by depositing a compound having an unsubstituted or monosubstituted amino group and a carbon compound on the substrate and then introducing a functional group capable of covalently binding to a nucleic acid molecule.

[0014]

(6) The solid support according to any one of the foregoing (1) to (4), which is obtained by dipping the substrate in a solution containing a compound having an unsubstituted or monosubstituted amino group and then introducing a functional group capable of

covalently binding to a nucleic acid molecule.

(7) The solid support according to the foregoing (6), in which the compound having an unsubstituted or monosubstituted amino group is polyarylamine.

(8) The solid support according to any one of the foregoing (1) to (7), in which the nucleic acid molecule is DNA.

substrate and then introducing a functional group capable of covalently binding to a nucleic acid molecule.

[0015]

(11) A method of producing a solid support characterized by dipping the substrate in a solution containing a compound having an unsubstituted or monosubstituted amino group and then introducing a functional group capable of covalently binding to a nucleic acid molecule.

(12) A method of immobilizing a primer on a solid support according to any one of (1) to (8), and hybridizing a nucleic acid molecule to the primer, thereby extending a nucleic acid molecule complementary to the nucleic acid molecule.

(13) A method of detecting a nucleic acid molecule, which comprises immobilizing a primer on a solid support according to any one of (1) to (8), hybridizing a nucleic acid molecule

to the primer, extending a nucleic acid molecule complementary to the nucleic acid molecule in the presence of a labeled nucleic acid and reading a signal derived from the labeled nucleic acid incorporated into the complementary nucleic acid molecule.

(14) A method of amplifying a nucleic acid molecule by immobilizing a primer on a solid support according to any one

(16) The method according to (13), which further comprises the step of amplifying the nucleic acid molecule after hybridizing a nucleic acid molecule to the primer.

[0016]

[Mode for carrying out the Invention]

Examples of a material of the substrate to be used in the present invention include, for example, silicon, glass, fiber, wood, paper, ceramics, and plastic (e.g., polyester resin, polyethylene resin, polypropylene resin, ABS resin (acrylonitrile butadiene styrene resin), nylon, acrylic resin, fluoro resin, polycarbonate resin, polyurethane resin, methylpentene resin, phenolic resin, melamine resin, epoxy resin, polyvinyl chloride resin).

[0017]

In the case where the foregoing members are used as the

material of the substrate, a surface-treated layer may not be provided, however, it is more preferred that surface treatment be carried out in order to firmly immobilize a compound for introducing a functional group capable of covalently binding to a nucleic acid molecule on the substrate.

[0018]

carbide, silicon carbide, tantalum carbide, thorium carbide, titanium carbide, uranium carbide, tungsten carbide, zirconium carbide, molybdenum carbide, chromium carbide or vanadium carbide may be used. The term of soft diamond here is used as a collective term of a partial diamond structure, which is a mixture of diamond and carbon, such as, so-called a diamond-like carbon (DLC), and the mixing ratio thereof is not particularly limited.

[0019]

As one example of the surface-treated substrate, a substrate in which a film has been formed with a soft diamond on a slide glass is exemplified. It is preferred that such a substrate be produced by the ionization deposition method in a mixed gas containing 0 to 99% by volume of hydrogen gas and the balance of methane gas (100 to 1% by volume) with a diamond-like

carbon.

[0020]

It is preferred that the thickness of the surface-treated layer be 1 nm to 100  $\mu\text{m}$ .

The formation of the surface-treated layer on the substrate can be carried out by a known method such as the microwave

method, arc deposition method or laser deposition method, for example.

[0021]

Examples of the substrate to be used in the present invention include, not only the structure in which the surface-treated layer has been formed as described above, but also synthetic diamond, high-pressure synthetic diamond, natural diamond, a soft diamond (e.g., a diamond-like carbon), amorphous carbon; metals such as gold, silver, copper, aluminum, tungsten and molybdenum; plastic (such as polyester resin, polyethylene resin, polypropylene resin, ABS resin, nylon, acrylic resin, fluorocarbon resin, polycarbonate resin, polyurethane resin, methylpentene resin, phenolic resin, melamine resin, epoxy resin, polyvinyl chloride resin); the one formed by mixing and combining powder of the foregoing metal,

powder of ceramic or the like with the foregoing resin as a binder; the one obtained by sintering at a high temperature a material such as powder of the foregoing metal or powder of ceramic, which has been powder-pressed with press-molding machine. In addition, the substrate may be a laminated product or a composite of the foregoing materials (for example, a composite of diamond and

one in the form of plate, it is generally about 0.1 to 100 mm of width, 0.1 to 100 mm of length and 0.01 to 10 mm of thickness.

[0023]

In addition, on the front face or back face of the substrate, a monolayer of Ti, Au, Pt, Nb, Cr, TiC, TiN or the like, or a composite layer thereof may be formed as a reflective layer. The thickness of the reflective layer is preferably 10 nm or more, more preferably 100 nm or more, because it is necessary to be uniform throughout the surface.

[0024]

In the case of using glass as the substrate, it is also preferred that the surface be intentionally roughened within the range of 1 nm to 1000 nm expressed in Ra (JIS B 0601). Such roughened surface is advantageous in that the surface area of the substrate is increased, whereby a large amount of DNA probes

or the like can be immobilized at a high density.

[0025]

The solid support of the present invention is provided with an electrostatic layer for electrostatically attracting a nucleic acid molecule.

The electrostatic layer is not particularly limited as

compound include a compound having an unsubstituted amino group (-NH<sub>2</sub>) or an amino group, which has been monosubstituted with an alkyl group having 1 to 6 carbon atoms or the like (-NHR; R is a substituent), and for example, ethylenediamine, hexamethylenediamine, n-propylamine, monomethylamine, dimethylamine, monoethylamine, diethylamine, arylamine, aminoazobenzene, aminoalcohol, (e.g., ethanolamine), acrinol, aminobenzoic acid, aminoanthraquinone, amino acids (glycine, alanine, valine, leucine, serine, threonine, cysteine, methionine, phenylalanine, tryptophan, tyrosine, proline, cystine, glutamic acid, aspartic acid, glutamine, asparagine, lysine, arginine and histidine), aniline, a polymer thereof (e.g., polyarylamine and polylysine) and a copolymer thereof; a polyamine (polyvalent amine) such as 4,4',4''-triaminotriphenylmethane, triamterene, spermidine,

spermin and putrescine.

[0027]

The electrostatic layer may be formed without covalently binding to the substrate or the surface-treated layer, or may be formed by covalently binding to the substrate or the surface-treated layer.

layer is formed. As the compound to be introduced into a film-forming apparatus, ammonia gas may be used. In addition, the surface-treated layer may be a multiple layer in which a film containing an amino group is formed after forming an adherent layer. Also in this case, film forming may be carried out also in an atmosphere containing ammonia gas.

[0029]

Further, in the case of forming the electrostatic layer without covalently binding to the substrate or the surface-treated layer, it is preferred to introduce a functional group capable of covalently binding to a nucleic acid molecule after depositing the forgoing compound having an unsubstituted or monosubstituted amino group and a carbon compound on the substrate in order to enhance the affinity, namely adhesiveness between the electrostatic layer and the substrate or the

surface-treated layer. The carbon compound to be used here is not particularly limited as long as it can be supplied as a gas, however, preferred are, for example, methane, ethane and propane that are gas at a normal temperature. As the method for deposition, the ionization deposition method is preferred. As the condition of the ionization deposition method, it is preferred that the

irradiating the substrate or the substrate provided with the surface-treated layer with ultraviolet rays in chlorine gas to chlorinate the surface, and reacting, among the foregoing amino group-containing compounds, for example, a polyvalent amine such as polyarylamine, polylysine 4,4',4''-triaminotriphenyl-methane or triamterene to introduce an amino group into the terminus to which the substrate does not bind.

[0031]

Further, in the case of carrying out the reaction of introducing a functional group capable of covalently binding to a nucleic acid molecule into the substrate provided with the electrostatic layer (e.g., introduction of a carboxyl group by using a dicarboxylic acid or polyvalent carboxylic acid) in a solution, it is preferred to introduce a functional group capable

of covalently binding to a nucleic acid molecule after dipping the substrate in a solution containing the foregoing compound having an unsubstituted or monosubstituted amino group. Examples of the solvent for the foregoing solution include, for example, water N-methylpyrrolidone and ethanol.

[0032]

[0033]

In the case of forming the electrostatic layer by dipping the substrate in a solution containing the compound having an unsubstituted or monosubstituted amino group, if polyarylamine is used as the amino group-containing compound, the adhesiveness to the substrate will be excellent and the immobilized amount of the nucleic acid molecule will be more improved.

[0034]

It is preferred that the thickness of the electrostatic layer be in the range of 1 nm to 500  $\mu$ m.

As described above, the surface of the substrate provided with the electrostatic layer is chemically modified in order to introduce a functional group capable of covalently binding to a nucleic acid molecule.

Examples of the foregoing functional group include, for

example, a carboxyl group, active ester group, haloformyl group, hydroxyl group, sulfate group, cyano group, nitro group, thiol group and amino group.

[0035]

Examples of the compound to be used for introducing a carboxyl group as the functional group include, for example,

dicarboxylic acid represented by the formula: HOOC-R<sup>2</sup>-COOH (wherein R<sup>2</sup> represents a single bond or a divalent hydrocarbon group having 1 to 12 carbon atoms) such as oxalic acid, malonic acid, succinic acid, maleic acid, fumaric acid or phthalic acid; a polyvalent carboxylic acid such as polyacrylic acid, polymethacrylic acid, trimellitic acid or butanetetracarboxylic acid; a keto acid or aldehyde acid represented by the formula: R<sup>3</sup>-CO-R<sup>4</sup>-COOH (wherein R<sup>3</sup> represents a hydrogen atom or a divalent hydrocarbon group having 1 to 12 carbon atoms and R<sup>4</sup> represents a divalent hydrocarbon group having 1 to 12 carbon atoms); a monohalide of dicarboxylic acid represented by the formula: X-OC-R<sup>5</sup>-COOH (wherein X represents a halogen atom and R<sup>5</sup> represents a single bond or a divalent hydrocarbon group having 1 to 12 carbon atoms) such as monochloride succinate or monochloride malonate; an acid

anhydride such as phthalic acid anhydride, succinic acid anhydride, oxalic acid anhydride, maleic acid anhydride or butanetetracarboxylic acid anhydride.

[0036]

With regard to the carboxyl group introduced as described above, active esterification thereof can be carried out with

a dihalide of dicarboxylic acid represented by the formula:  
 $X-OC-R^6-CO-X$  (wherein X represents a halogen atom, and  $R^6$  represents a single bond or a divalent hydrocarbon group having 1 to 12 carbon atoms) such as chloride succinate or chloride malonate.

[0038]

Examples of the compound to be used for introducing a hydroxyl group as the functional group include, for example, a hydroxy acid or phenol acid represented by the formula:  
 $HO-R^7-COOH$  (wherein  $R^7$  represents a divalent hydrocarbon group having 1 to 12 carbon atoms).

[0039]

Examples of the compound to be used for introducing an amino group as the functional group include, for example, an amino acid.

The foregoing compound forms an amide bond by condensing the carboxyl group with the amino group in the electrostatic layer.

[0040]

Among the foregoing compounds, the polyvalent carboxylic acids such as polyacrylic acid, polymethacrylic acid, trimellitic

5 to 150. In addition, in the case of DNA, either a single-stranded or double-stranded chain can be immobilized.

[0042]

The solid support of the present invention can be used for an extension reaction of a nucleic acid molecule such as DNA. In this case, first a primer is immobilized on the solid support, and then a single-stranded or double-stranded DNA is hybridized. Thereafter, DNA complementary to the DNA hybridized to the primer is extended by a DNA extension reaction.

[0043]

As the primer, a single-stranded or double-stranded nucleic acid molecule whose length and sequence are known is used. The length is not particularly limited, however, it is preferably 5 to 200 bases, more preferably 10 to 100 bases. The method of immobilizing the primer is not particularly limited,

however, for example, a primer solution is prepared by dissolving a nucleic acid molecule in a buffer and the solid support of the present invention is dipped in the solution, whereby the primer can be immobilized on the surface of the solid support. The primer can be immobilized by performing dipping at generally 0 to 98C°, preferably 4 to 50C° for generally 1 minute to 24

after primer solutions are spotted on the solid support with a spotter, baking is performed in a heated oven for a predetermined period of time, thereafter the washing is performed, whereby the primer that is not immobilized is removed. By using the spotter, a wide variety of primers can be immobilized at different positions on the solid support, whereby a large number of assays can be carried out at one time, therefore it is advantageous in the field of detecting nucleic acids, which requires enormous assays.

[0044]

In the conventional solid support, the primer is peeled off by a heat treatment in an extension reaction in some cases. On the other hand, in the solid support of the present invention, the primer is not peeled off even if heat is applied, and an extension reaction can be carried out in the state in which the

primer has been immobilized on the solid support.

[0045]

During this extension reaction, a labeled nucleic acid is allowed to be incorporated, and after the extension reaction, by reading a signal derived from the label, it can be detected whether or not a specific DNA has been hybridized to the primer

The label is not particularly limited as long as it can be incorporated into a nucleic acid molecule, however, examples include a fluorescent label (CyDye such as Cy3 and Cy5, FITC, RITC, Rhodamine, TexasRed, TET, TAMRA, FAM, HEX, ROX and the like), a radiolabel ( $\alpha$ - $^{32}$ P,  $\gamma$ - $^{32}$ P,  $^{35}$ S and the like) etc, for example. In the case of using the fluorescence-labeled nucleic acid, it can be detected by making a fluorescent photograph of the solid support after the extension reaction.

[0047]

The solid support of the present invention can be used in DNA amplification reaction. In the case of amplifying it by PCR reaction, for example, first a forward primer is immobilized on the solid support, a single-stranded or double-stranded DNA is hybridized, and then a complementary strand DNA is extended by an enzymatic reaction. Further, by carrying out the steps

of (1) annealing, (2) hybridization and (3) extension reaction continuously, so-called PCR reaction progresses.

[0048]

In the conventional solid support, there was a problem that the primer is peeled off by a heat treatment in PCR reaction or the control of thermal cycle could not be carried out well.

nucleotide sequence to be amplified, or to replicate DNA other than the target, therefore, DNA can be amplified efficiently.

[0049]

In the case of using the solid support of the present invention for DNA amplification by the foregoing PCR, it is preferred that the thermal conductivity of the solid support be 0.1 W/cmK or higher, more preferably 0.5 W/cmK or higher, most preferably 1 W/cmK or higher. It is based on the fact that if the thermal conductivity of the solid support is high, the following property for heating and cooling is superior in the case of carrying out PCR reaction or the like.

[0050]

Specifically, it is preferred that as the substrate for producing the solid support, in terms of the thermal conductivity, diamond or the one coated with diamond or the like as the

surface-treated layer on a variety of substrates be used.

[0051]

Further, by combining the foregoing detection using the labeled nucleic acid with amplification by PCR, even in the case where only a small amount of DNA which can be hybridized to the primer on the solid support is contained in a sample, DNA is

reaction, a strand-displacing DNA polymerase is selected and a reverse primer is added, whereby DNA can be amplified on the solid support at a constant temperature without undergoing the thermal cycle. The strand-displacing DNA polymerase is a DNA synthase that can synthesize the complementary strand continuously by dissociating the double-stranded region if the double-stranded region is present in the extending direction during the process of synthesizing a DNA strand complementary to a template DNA.

[0053]

The strand-displacing DNA polymerase is not particularly limited, however, examples include, for example, Bca BEST DNA polymerase (Takara Bio), Phi29 DNA polymerase (Amersham Bioscience) and the like.

[0054]

In another example of the present invention, by using cDNA synthesized from mRNA as a target, RNA, though it is indirect, can also become a target.

[0055]

In this case, cDNA is obtained from mRNA by utilizing a reverse transcription reaction, however, cDNA can be

comprising a sequence, in which there are about 10 to 20 Ts (thymines) in a row, corresponding to the poly(A)<sup>+</sup> sequence at the 5'-terminus of RNA.

[0056]

In the case of using the oligo dT primer, the poly(A) region at the 5'-terminus of RNA to be used as the template is annealed. A reverse transcriptase is allowed to act on this, and dNTP complementary to the template RNA is polymerized at the 3'-terminus of the primer one after another, whereby cDNA is synthesized in the direction from the 5' to the 3'. The binding of primer, annealing, and polymerization of a complementary strand by a reverse transcriptase in this reverse transcription reaction can be carried out by controlling the temperature (thermal cycle) according to the usual methods.

[0057]

As described above, immobilization on the solid support can be carried out at the same time when reverse transcription reaction is carried out, therefore, according to the method of the present invention, so-called RT-PCR (reverse transcript-PCR) can be efficiently carried out. Therefore, the present invention is also useful for quantifying mRNA.

resultant product can be also used as a DNA library chip. In addition, by immobilizing a nucleotide, oligonucleotide, DNA fragment or the like instead of DNA, the resultant product can be also used as a library.

[0059]

By performing the detection as described above with the use of the solid support of the present invention, diagnosis of a disease can be also performed.

[0060]

[Examples]

Hereunder, the present invention will be explained with reference to the examples, however, the present invention is not intended to be limited thereto.

(Example 1)

Introduction of Amino Group-containing Compound into

Chamber when Applying Surface-treated Layer to Substrate (1)

A DLC layer was formed at a thickness of 10 nm on a slide glass of 25 mm (width) × 75 mm (length) × 1 mm (thickness) by the ionization deposition method, at an accelerating voltage of 0.5 kV, using a mixed gas of 95% by volume of methane gas and 5% by volume of hydrogen gas as material. Then, using methane gas

Then, after butanetetracarboxylic acid anhydride, as a polyvalent carboxylic acid, was condensed with the amino group in the surface-treated layer consisting of C, N and H formed with methane and ethylenediamine as material, it was activated by being dipped in an activation solution, in which 0.1 M 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide and 20 mM N-hydroxysuccinimid had been dissolved in 300 ml of 0.1 M phosphate buffer (pH 6), for 30 minutes.

[0062]

Then, About 1 nl of 500 bp Cy3-labeled double-stranded DNA, which had been amplified by PCR using λDNA prepared at a concentration of 0.1 µg/µl as a template, was spotted on the substrate using a microarray maker. Then, after it was heated in an oven at 80°C for 3 hours and washed with 2×SSC/0.2% SDS, the intensity of fluorescence of the spotted DNA was measured.

[0063]

As a result, the intensity of fluorescence was 36,050. Further, when the intensity of fluorescence was measured after performing washing with 2×SSC/0.2% SDS at 95C°, it was 35,540, which was hardly decreased at all.

[0064]

by being dipped in an activation solution, in which 0.1 M 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide and 20 mM N-hydroxysuccinimidate had been dissolved in 300 ml of 0.1 M phosphate buffer (pH 6), for 30 minutes. After 500 bp Cy3-labeled double-stranded DNA, which had been amplified by PCR using λDNA as a template, was immobilized in the same manner on the thus obtained substrate, the substrate was washed with 2×SSC/0.2% SDS. As a result, the intensity of fluorescence was 23,500. Further, when the intensity of fluorescence was measured after performing washing with 2×SSC/0.2% SDS at 95C°, it was 23,000, which was hardly decreased at all.

[0065]

In other word, by using a covalent bond type substrate that hardly has an electrostatic layer, although DNA can be immobilized more firmly by a covalent bond, the intensity of

a fluorescence signal was not increased.

[0066]

Further, after 500 bp Cy3-labeled double-stranded DNA, which had been amplified by PCR using  $\lambda$ DNA as a template, was immobilized in the same manner on a substrate that does not have an electrostatic layer (after 5% polyacrylic acid aqueous

was washed with 2 $\times$ SSC/0.2% SDS. As a result, though the polyacrylic acid film was peeled off, the intensity of fluorescence was 26,220 in the region where the layer remained. Further, the polyacrylic acid film was completely peeled off when being washed with 2 $\times$ SSC/0.2% SDS at 95C°.

[0067]

(Example 2)

Introduction of Amino Group-containing Compound into Chamber when Applying Surface-treated Layer to Substrate (2)

To a slide glass of 25 mm (width)  $\times$ 75 mm (length)  $\times$ 1 mm (thickness) by the ionization deposition method, using methane gas as a carrier gas, at the rate of 5 cm $^3$ /minute, it was introduced into a chamber through the ethylenediamine incubated at 15C°. At a working pressure of 2 Pa and an accelerating voltage of 0.5 kv, using methane and ethylenediamine as material, a layer

consisting of C, N and H was formed at a thickness of 20 nm.

[0068]

Then, after polyacrylic acid, as a polyvalent carboxylic acid, was condensed with the amino group in the surface-treated layer consisting of methane and ethylenediamine in the presence of 0.1 M 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide, it

DNA, which had been amplified by PCR using  $\lambda$  DNA prepared at a concentration of 0.1 $\mu$ g/ $\mu$ l as a template, was spotted on the substrate using a microarray maker. Then, after it was heated in an oven at 80°C for 3 hours and washed with 2 $\times$ SSC/0.2% SDS, the intensity of fluorescence of the spotted DNA was measured.

[0070]

As a result, the intensity of fluorescence was 34,050. Further, when the intensity of fluorescence was measured after performing washing with 2 $\times$ SSC/0.2% SDS at 95°C, it was 33,500, which was hardly decreased at all.

[0071]

As a comparison, after 500 bp Cy3-labeled double-stranded DNA, which had been amplified by PCR using  $\lambda$  DNA as a template, was immobilized in the same manner on a commercially available electrostatic type substrate (manufactured by Matsunami Glass

Ind., LTD.; a substrate which is a slide glass applied with aminosilane (a silane coupling agent)), the substrate was washed with 2×SSC/0.2% SDS. As a result, the intensity of fluorescence was 35,460. Further, when the intensity of fluorescence was measured after performing washing with 2×SSC/0.2% SDS at 95C°, it was decreased to 26,210.

rays were irradiated for 60 minutes to make it insoluble. Then, it was activated by being dipped in an activation solution, in which 0.1 M 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide and 20 mM N-hydroxysuccinimide had been dissolved in 300 ml of 0.1 M phosphate buffer (pH 6), for 30 minutes), the substrate was washed with 2×SSC/0.2% SDS. As a result, though the polyacrylic acid film was peeled off, the intensity of fluorescence was 26,220 in the region where the layer remained. Further, the polyacrylic acid film was completely peeled off when being washed with 2×SSC/0.2% SDS at 95C°.

[0073]

(Example 3)

#### Formation of Electrostatic Layer by Post-treatment

A DLC layer was formed at a thickness of 10 nm on a slide glass of 25 mm (width) ×75 mm (length) ×1 mm (thickness) by the

ionization deposition method, at an accelerating voltage of 0.5 kV, using a mixed gas of 95% by volume of methane gas and 5% by volume of hydrogen gas as material.

Then, it was chlorinated by being irradiated with ultraviolet rays for 30 minutes in chlorine gas. Then, the substrate was dipped in a polyacrylic amine aqueous solution

activated by being dipped in an activation solution, in which 0.1 M 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide and 20 mM N-hydroxysuccinimide had been dissolved in 300 ml of 0.1 M phosphate buffer (pH 6), for 30 minutes.

[0075]

Then, about 1 nl of 500 bp Cy3-labeled double-stranded DNA, which had been amplified by PCR using  $\lambda$ DNA prepared at a concentration of 0.1 $\mu$ g/ $\mu$ l as a template, was spotted on the substrate using a microarray maker. Then, after it was heated in an oven at 80°C for 3 hours and washed with 2 $\times$ SSC/0.2% SDS, the intensity of fluorescence of the spotted DNA was measured.

[0076]

As a result, the intensity of fluorescence was 35,000. Further, when the intensity of fluorescence was measured after performing washing with 2 $\times$ SSC/0.2% SDS at 95°C, it was 34,500,

which was hardly decreased at all.

[0077]

As a comparison, after 5% polyacrylic acid aqueous solution was applied to a slide glass on which DLC was formed at a thickness of 10 nm and dried, ultraviolet rays were irradiated for 60 minutes to make it insoluble. Then, it was activated by

with 2×SSC/0.2% SDS, the polyacrylic acid film was completely peeled off.

[0078]

(Example 4)

#### Immobilization of Primer by Dipping Method

A DLC layer was formed at a thickness of 100 nm on an Si substrate cut into 3 mm square by the ionization deposition method, at an accelerating voltage of 0.5 kv, using a mixed gas of 95% by volume of methane gas and 5% by volume of hydrogen gas as material. Then, methane gas and hydrogen gas were replaced with ammonia gas atmosphere and aminization was carried out for 10 minutes by the plasma method.

[0079]

Then, after a polyvalent carboxylic acid (polyacrylic acid) was condensed with the amino group introduced in the

surface-treated layer, it was activated by being dipped in an activation solution, in which 0.1 M 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide and 20 mM N-hydroxysuccinimid had been dissolved in 0.1 M phosphate buffer (pH 6), for 30 minutes.

[0080]

[0081]

The solid support, on which the forward primer had been immobilized, was dipped in a solution of the 500 bp λDNA that is complementary to the immobilized primer (0.025 µg/µl, buffer: 5×SSC/0.5% SDS/20% formamide). After it was incubated at 98°C° for 5 minutes, it was incubated at 42°C° for 12 hours. After the reaction, the solid support was washed with a washing solution (2×SSC/0.2% SDS) twice and rinsed with sterile water.

[0082]

The solid support after hybridization was dipped in a reaction solution (1×Exbuffer/0.025mM Cy3-dCTP/1.25mM dNTP/0.25 U ExTaq) and incubated at 42°C° for 6 hours. After the reaction, the solid support was washed with a washing solution (2×SSC/0.2% SDS) twice and rinsed with sterile water.

When the solid support after the washing was observed

with a fluorescence image scanner, a fluorescence signal by the extension reaction was detected.

[0083]

(Example 5)

#### Immobilization of Primer by Spotting Method (1)

A DLC layer was formed at a thickness of 100 nm on an

[0084]

Then, after a polyvalent carboxylic acid (polyacrylic acid) was condensed with the amino group introduced in the surface-treated layer, it was activated by being dipped in an activation solution, in which 0.1 M 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide and 20 mM N-hydroxysuccinimide had been dissolved in 0.1 M phosphate buffer (pH 6), for 30 minutes.

[0085]

Then, solutions of forward primers (22 bases, 10 samples) of the 500 bp λDNA prepared at a concentration of 0.1 $\mu$ g/ $\mu$ l using 20% DMSO solution were spotted on the solid support by using a spotter. Then, after being placed in an oven heated at 80°C for 1 hour, it was washed with a washing solution (2×SSC/0.2% SDS) twice and rinsed with sterile water.

[0086]

The solid support, on which the primers had been immobilized, was dipped in a solution of the single-stranded 500 bp  $\lambda$  DNA that is complementary to the immobilized primers (0.025 $\mu$ g/ $\mu$ l, buffer: 5 $\times$ SSC/0.5% SDS/20% formamide). After it was incubated at 98°C for 5 minutes, it was incubated at 42°C

dNTP/0.25 U ExTaq) and incubated at 42°C for 6 hours. After the reaction, the solid support was washed with a washing solution (2 $\times$ SSC/0.2% SDS) twice and rinsed with sterile water.

When the solid support after the washing was observed with a fluorescence image scanner, a fluorescence signal by the extension reaction was detected.

[0088]

(Example 6)

#### Immobilization of Primer by Spotting Method (2)

A DLC layer was formed at a thickness of 10 nm on a slide glass of 25 mm (width)  $\times$  75 mm (length)  $\times$  1 mm (thickness) by the ionization deposition method, at an accelerating voltage of 0.5 kv, using a mixed gas of 95% by volume of methane gas and 5% by volume of hydrogen gas as material. Then, methane gas and hydrogen gas were replaced with ammonia gas atmosphere and

aminization was carried out for 10 minutes by the plasma method.

[0089]

Then, after a polyvalent carboxylic acid (polyacrylic acid) was condensed with the amino group introduced in the surface-treated layer, it was activated by being dipped in an activation solution, in which 0.1 M

20% DMSO solution were spotted on the solid support by using a spotter. Then, after being placed in an oven heated at 80°C for 1 hour, it was washed with a washing solution (2×SSC/0.2% SDS) twice and rinsed with sterile water.

[0091]

On the solid support, 15μL of a solution of the 500 bp λDNA that is complementary to the immobilized primers (0.025μg/μL, buffer: 5×SSC/0.5% SDS/20% formamide) was placed and further overlaid with a cover glass. After hybridization was carried out by incubating at 42°C for 5 hours, the cover glass was washed out with 0.1×SSC and the solid support was washed with a washing solution (2×SSC/0.2% SDS) twice and rinsed with sterile water.

[0092]

On the solid support after hybridization, 15μl of a reaction solution (1×Exbuffer/0.025 mM Cy3-dCTP/1.25 mM

dNTP/0.25 U ExTaq) was placed and further overlaid with a cover glass. After an extension reaction was carried out by incubating at 42C° for 5 hours, the cover glass was washed out with 0.1×SSC and the solid support was washed with a washing solution (2×SSC/0.2% SDS) twice and rinsed with sterile water.

When the solid support after the washing was observed

Si substrate cut into 3 mm square by the ionization deposition method, at an accelerating voltage of 0.5 kv, using a mixed gas of 95% by volume of methane gas and 5% by volume of hydrogen gas as material. Then, methane gas and hydrogen gas were replaced with ammonia gas atmosphere and aminization was carried out for 10 minutes by the plasma method.

[0094]

Then, after a polyvalent carboxylic acid (polyacrylic acid) was condensed with the amino group introduced in the surface-treated layer, it was activated by being dipped in an activation solution, in which 0.1 M 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide and 20 mM N-hydroxysuccinimide had been dissolved in 0.1 M phosphate buffer (pH 6), for 30 minutes.

[0095]

Then, solutions of forward primers (22 bases, 10 samples) of the 500 bp  $\lambda$ DNA prepared at a concentration of 0.1 $\mu$ g/ $\mu$ l using 20% DMSO solution were spotted on the solid support by using a spotter. Then, after being placed in an oven heated at 80°C for 1 hour, it was washed with a washing solution (2 $\times$ SSC/0.2% SDS) twice and rinsed with sterile water.

The reaction solution was placed in a PCR tube and further a reverse primer was added. The solid support, on which the primers had been immobilized, was dipped in the reaction solution and a cycle consisting of annealing (94°C, 1 minute), hybridization (60°C, 1 minute) and extension reaction (72°C, 1 minute) was repeated 30 times. After the reaction, the solid support was washed with a washing solution (2 $\times$ SSC/0.2% SDS) twice and rinsed with sterile water.

[0098]

When the solid support after the washing was observed with a fluorescence image scanner, a more intense fluorescence signal was detected than in the case where the reaction was carried out at a constant temperature.

Further, when the solid support, with which the extension reaction had been carried out, was put in a PCR reaction solution

and PCR reaction was carried out to amplify the 500 bp  $\lambda$ DNA, the amplification of 500 bp region was confirmed by electrophoresis.

[0099]

(Example 8)

#### Amplification Reaction Using Strand-displacing DNA

with ammonia gas atmosphere and aminization was carried out for 10 minutes by the plasma method.

[0100]

Then, after a polyvalent carboxylic acid (polyacrylic acid) was condensed with the amino group introduced in the surface-treated layer, it was activated by being dipped in an activation solution, in which 0.1 M 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide and 20 mM N-hydroxysuccinimide had been dissolved in 0.1 M phosphate buffer (pH 6), for 30 minutes.

[0101]

Then, solutions of forward primers (22 bases, 10 samples) of the 500 bp  $\lambda$ DNA prepared at a concentration of 0.1 $\mu$ g/ $\mu$ l using 20% DMSO solution were spotted on the solid support by using a spotter. Then, after being placed in an oven heated at 80C°

for 1 hour, it was washed with a washing solution (2×SSC/0.2% SDS) twice and rinsed with sterile water.

[0102]

The solid support, on which the primers had been immobilized, was dipped in a solution of the 500 bp  $\lambda$ DNA that is complementary to the immobilized primers (0.025 $\mu$ g/ $\mu$ l, buffer:

reaction solution (BcaBEST DNA polymerase/20 mM Tris/10 mM MgCl<sub>2</sub>) , to which a reverse primer had been added, and was incubated at 60°C for 6 hours. After the reaction, the solid support was washed with a washing solution (2×SSC/0.2% SDS) twice and rinsed with sterile water. The enzyme used is a strand-displacing DNA polymerase (manufactured by TAKARA) .

[0104]

When the solid support after the washing was observed with a fluorescence image scanner, a fluorescence signal by the extension reaction was detected.

Further, when the solid support, with which the extension reaction had been carried out, was put in a PCR reaction solution and PCR reaction was carried out to amplify the 500 bp  $\lambda$ DNA, the amplification of 500 bp region was confirmed by electrophoresis.

[0105]

(Example 9)

Annealing, Hybridization, Extension Reaction (2)

A DLC layer was formed at a thickness of 100 nm on an Si substrate cut into 3 mm square by the ionization deposition method, at an accelerating voltage of 0.5 kv, using a mixed gas

- - -  
acid) was condensed with the amino group introduced in the surface-treated layer, it was activated by being dipped in an activation solution, in which 0.1 M 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide and 20 mM N-hydroxysuccinimide had been dissolved in 0.1 M phosphate buffer (pH 6), for 30 minutes.

[0107]

Then, a 0.1 $\mu$ g/ $\mu$ l forward primer solution (5'-GATGAGTTGTGTCCGTACAAC-3', 22bases, 20% DMSO), a 0.1 $\mu$ g/ $\mu$ l reverse primer solution (5'-GGTTATCGAAATCAGCCACAGCGCC-3', 20% DMSO) and a 0.05 $\mu$ g/ $\mu$ l forward+reverse primer solution (20% DMSO) were spotted on the solid support by using a spotter. Then, after being placed in an oven heated at 80°C for 1 hour, it was washed with a washing solution (2 $\times$ SSC/0.2% SDS) twice and rinsed with sterile water.

[0108]

Reaction solution 1 (0.025 $\mu$ g/ $\mu$ l  $\lambda$ DNA/1 pmol/ $\mu$ l reverse primer/1 $\times$ Exbuffer/0.025 mM Cy3-dCTP/1.25 mM dNTP/0.25U ExTaq) was placed in a PCR tube and the solid support, on which the primers had been immobilized, was dipped in the reaction solution and a cycle consisting of annealing (94C°, 30 seconds),

with a fluorescence image scanner, a more intense fluorescence signal was detected than in the case where the reaction was carried out at a constant temperature.

Further, when the solid support, with which the extension reaction had been carried out, was put in reaction solution 2 (1 pmol/ $\mu$ l forward primer/1 pmol/ $\mu$ l reverse primer/1 $\times$ Exbuffer/1.25 mM dNTP/0.25 U ExTaq) and a cycle consisting of annealing (94C°, 30 seconds), hybridization and extension reaction (68C°, 30 seconds) was repeated 30 times, the amplification of DNA fragment in the 500 bp region was confirmed by electrophoresis.

[0110]

(Example 10)

#### Immobilization of Primer by Dipping Method (2)

After a glass substrate cut into 3 mm square was dipped

in a polyacrylic amine aqueous solution adjusted to 0.1% and a polyvalent carboxylic acid (polyacrylic acid) was condensed with the amino group introduced in the substrate surface, it was activated by being dipped in an activation solution, in which 0.1 M 1-[3-(dimethylamino) propyl]-3-ethylcarbodiimide and 20 mM N-hydroxysuccinimidate had been dissolved in 0.1 M phosphate

reaction, the solid support was washed with a washing solution (2×SSC/0.2% SDS) twice and rinsed with sterile water.

[0112]

The solid support, on which the forward primer had been immobilized, was dipped in a solution of the 500 bp λDNA that is complementary to the immobilized primer (0.025μg/μl, buffer: 5×SSC/0.5% SDS/20% formamide). After it was incubated at 98C° for 5 minutes, it was incubated at 42C° for 12 hours. After the reaction, the solid support was washed with a washing solution (2×SSC/0.2% SDS) twice and rinsed with sterile water.

[0113]

The solid support after hybridization was dipped in a reaction solution (1×Exbuffer/0.025 mM Cy3-dCTP/1.25 mM dNTP/0.25 U ExTaq) and incubated at 42C° for 6 hours. After the reaction, the solid support was washed with a washing solution

(2×SSC/0.2% SDS) twice and rinsed with sterile water.

When the solid support after the washing was observed with a fluorescence image scanner, a fluorescence signal by the extension reaction was detected.

[0114]

(Comparative example)

1 hour in a solution of a forward primer (22 bases) of the 500 bp λDNA prepared at a concentration of 0.1 $\mu$ g/ $\mu$ l using sterile water, the immobilization reaction by electrostatic binding was carried out. After the reaction, the substrate was washed with a washing solution (2×SSC/0.2% SDS) twice and rinsed with sterile water. In addition, a substrate that had been dipped in a solution of a fluorescence-labeled forward primer (22 bases) of the 500 bp λDNA was also prepared.

[0116]

The substrate, on which the forward primer had been immobilized, was dipped in a solution of the 500 bp λDNA that is complementary to the immobilized primer (0.025 $\mu$ g/ $\mu$ l, buffer: 5×SSC/0.5% SDS/20% formamide). After it was incubated at 98°C for 5 minutes, it was incubated at 42°C for 12 hours. After the reaction, the substrate was washed with a washing solution

(2×SSC/0.2% SDS) twice and rinsed with sterile water.

[0117]

The substrate after hybridization was dipped in a reaction solution (1×Exbuffer/0.025 mM Cy3-dCTP/1.25 mM dNTP/0.25 U ExTaq) and incubated at 42 C° for 6 hours. After the reaction, the substrate was washed with a washing solution (2×SSC/0.2%

fluorescence-labeled forward primer solution (22 bases) of the 500 bp λDNA had been immobilized, although a faint fluorescence signal was observed after the immobilization reaction, a fluorescence signal was not detected at all in the observation of a fluorescence image after the same procedure was carried out. This indicates that the primer immobilized on the substrate was peeled off during the procedure.

[0119]

The intensities of fluorescence measured with a fluorescence image scanner in the foregoing Examples 4 to 10 are summarized in the following Table 1.

[0120]

[Table 1]

Example	Intensity of Fluorescence Signal		
	Before reaction	Spot A	Spot B

4	4500	9000	-
5	4500	9500	11000
6	2500	6000	7500
7	4500	21000	22500
8	4500	15500	16000
9	4500	20000	22000
10	2000	7000	-

[0121]

array can be improved. Further, since an extension reaction can be performed in the state in which a nucleic acid molecule has been immobilized, or a nucleic acid molecule can be amplified by performing PCR reaction, it is possible to aim at generalizing a DNA array.

[Designation of Document] Abstract

[Abstract]

[Task]

To provide a solid support capable of immobilizing nucleic acid molecules in a high proportion and with a higher bond strength to the nucleic acid molecules.